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# Energy transduction in lactic acid bacteria

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**Abstract:** In the discovery of some general principles of energy transduction, lactic acid bacteria have played an important role. In this review, the energy transducing processes of lactic acid bacteria are discussed with the emphasis on the major developments of the past 5 years. This work not only includes the biochemistry of the enzymes and the bioenergetics of the processes, but also the genetics of the genes encoding the energy transducing proteins. The progress in the area of carbohydrate transport and metabolism is presented first. Sugar translocation involving ATP-driven transport, ion-linked cotransport, heterologous exchange and group translocation are discussed. The coupling of precursor uptake to product excretion and the linkage of antiport mechanisms to the deiminase pathways of lactic acid bacteria is dealt with in the second section. The third topic relates to metabolic energy conservation by chemiosmotic processes. There is increasing evidence that precursor/product exchange in combination with precursor decarboxylation allows bacteria to generate additional metabolic energy. In the final section transport of nutrients and ions as well as mechanisms to excrete undesirable (toxic) compounds from the cells are discussed.

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**Key words:** Energy transduction; Transport; Bioenergetics; Lactic acid bacteria

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## Introduction

Lactic acid bacteria metabolize (ferment) substrates without net production of reducing equivalents. As an example may serve the degradation of glucose to lactic acid, resulting in the production of 2 mol of ATP per mol of glucose metabolized. Some lactic acid bacteria, however, have limited capabilities to utilize exogenous electron acceptors for the degradation (oxidation) of substrates. Carbohydrate metabolism in *Lactococcus lactis* and *Streptococcus thermophilus* can be affected by the presence of oxygen. Under aerobic conditions and slow fermentation rates, *L. lactis*

is able to convert lactose almost quantitatively to acetic acid (plus carbon dioxide), thereby producing an excess of NADH [1]. These facultative anaerobic lactic acid bacteria contain flavin-type NADH oxidases and NADH peroxidase which obviates the need for pyruvate reduction to lactate or reduction of acetyl-CoA to ethanol. By forming acetic acid instead of lactic acid, and if an oxidase is used to reoxidize the NADH produced, additional ATP can be formed by substrate level phosphorylation. Theoretically, carbohydrate metabolism by lactic acid bacteria may also yield acetic acid as end product, and thus extra ATP, under anaerobic conditions if fumarate reductase is used. In this scheme electrons are transferred from a NADH dehydrogenase via (mena)quinones to fumarate reductase. All these components are present in the cytoplasmic membrane of *L. lactis* [2], but the use of this

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electron transfer chain in the oxidation of NADH under anaerobic conditions has never been demonstrated.

Since large quantities of fermentation products are formed in the cytoplasm of lactic acid bacteria, the outwardly directed concentration gradients of these molecules may become high. When these end-product gradients exceed the electrochemical proton (or sodium ion) gradient, and product efflux occurs carrier-mediated in symport with protons or sodium ions, additional metabolic energy can be generated by this process [3,4]. Metabolic energy will thus be conserved by transforming a product gradient into a proton (or sodium ion) gradient which, in turn, can be used to synthesize ATP via the  $F_0F_1$ -ATPase or to drive other endergonic processes.

In addition to the ATP produced in the glycolytic pathway, some lactic acid bacteria generate additional ATP by arginine or agmatine metabolism via the corresponding deiminase pathways [5–8].

Recently, another mechanism for the generation of metabolic energy in lactic acid bacteria has been discovered. Rather than forming ATP by substrate level phosphorylation, a proton motive force ( $\Delta p$ ) is formed by the combined action of a secondary transport mechanism responsible for the uptake of substrate and excretion of product and a decarboxylation reaction [9–11].

Energy transduction not only refers to processes that lead to the production of metabolic energy in the form ATP or ion concentration gradients, but also covers the various transport processes that form solute concentration gradients at the expense of metabolic energy. Solute transport systems in bacteria can be subdivided into three distinct mechanisms that differ in the form of energy that is used and the way this energy is transduced in the translocation process [10], i.e. primary (light, chemical energy), secondary (chemiosmotic energy) and group translocation transport (chemical modification concomitant with transport). Primary transport in lactic acid bacteria is exemplified by the ion- and solute-ATPases which utilize the change in free energy upon hydrolysis of ATP (to ADP plus inorganic phosphate) to transport solutes against

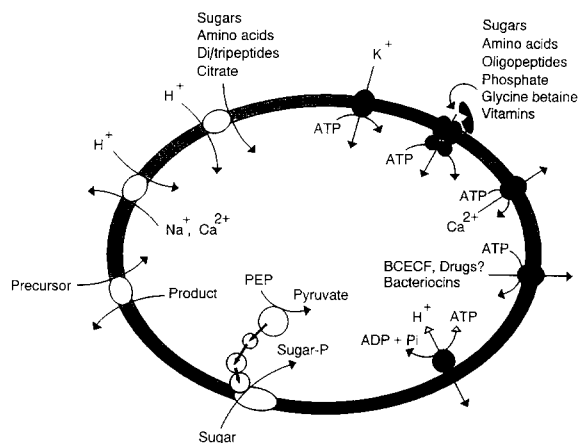


Fig. 1. Solute transport mechanisms in lactic acid bacteria. Primary (ATP-driven) transport systems are indicated as filled icons; secondary symport and antiport systems are indicated as open icons; the PEP-dependent sugar:phosphotransferase system is indicated by shaded icons. The examples given are primarily based on experimental evidence from work on *L. lactis* (see Table 2).

their concentration gradients (Fig. 1, filled icons). Secondary transport includes the ion-linked solute symport and antiport systems as well as the precursor/product exchange systems. These transport systems couple the transport of a solute to the (downhill) movement of a second molecule (often  $H^+$  or  $Na^+$ , or another compound that is structurally related to the substrate) (Fig. 1, open icons). Solutes (e.g. substrates, nutrients) can be concentrated in the cytoplasm by means of solute-cation symport, while solute/cation antiport systems allow the bacteria to keep the cytoplasmic concentration of certain solutes (e.g. unwanted products) below the medium concentration (Fig. 1, open icons). Group translocation in (lactic acid) bacteria has only been observed for carbohydrates and involves phosphorylation of the sugar concomitant with transport by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (Fig. 1, shaded icons). The subdivision of transport systems on basis of their mechanism of energy coupling will be followed throughout this manuscript. The first part of the review focusses on those transport processes that are linked to the pathways of metabolic energy generation, i.e. glycolytic, deiminase and solute decarboxylation

pathways. The PTS that forms one mechanism by which sugars can be transported into the cell will only be dealt with briefly (details on the PTS of Gram-positive bacteria are given in the review of Hengstenberg et al., this issue). In the second part of the review the nutrient and ion transporters, and some new excretion mechanisms of lactic acid bacteria will be discussed.

### Sugar transport and initial metabolism

The initial event in the metabolism of mono- and disaccharides involves the translocation of these molecules across the cytoplasmic membrane. The systems by which the carbohydrate molecules are transported can be subdivided into (i) sugar transport ATPases, (ii) ion-linked sugar transport and sugar exchange mechanisms, and (iii) PEP:sugar PTS. These different mechanisms of transport have been observed in lactic acid bacteria for a wide variety of sugars but those that mediate lactose (galactoside) transport have been described in greatest detail.

Depending on the transport mechanism the following step in lactose metabolism involves either hydrolysis of the free sugar by  $\beta$ -galactosidase (LacZ), yielding glucose and galactose, or hydrolysis of lactose 6-phosphate by phospho- $\beta$ -galactosidase (LacG), yielding glucose and galactose 6-phosphate. The two major pathways of glucose degradation in lactic acid bacteria are the glycolytic (or Embden-Meyerhof-Parnas) and the phosphoketolase pathway [12]. The glycolytic pathway is operative in *Lactococcus* spp., homofermentative lactobacilli (e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *helveticus*), pediococci and *Streptococcus thermophilus*, whereas the phosphoketolase pathway(s) resulting in heterofermentation is present in obligate heterofermentative species like *Leuconostoc* spp., *Lactobacillus buchneri* and *Lactobacillus brevis* [12,13]. In facultative heterofermentative species, like *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus pentosus*, both the glycolytic and phosphoketolase pathways can be present [13].

Galactose is metabolized further via the Leloir pathway (if not excreted into the medium, see

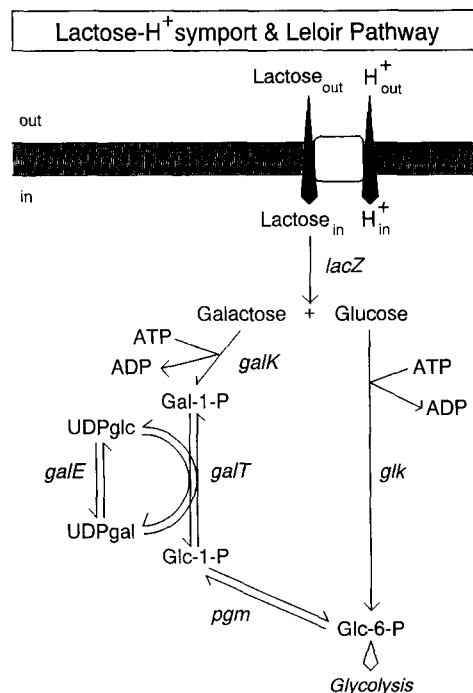


Fig. 2. Lactose-H<sup>+</sup> symport and Leloir pathway. *lacZ*,  $\beta$ -galactosidase; *galK*, galactokinase; *galT*, UDPglucose:galactose 1-phosphate uridylyl transferase; *galE*, UDPglucose 4-epimerase; *pgm*, phosphoglucomutase; *glk*, glucokinase.

below), whereas galactose 6-phosphate enters the tagatose 6-phosphate pathway. Similarly, other carbohydrates may enter the cell either as free sugar or phosphorylated derivative, and these molecules can be degraded further following phosphorylation by a kinase or directly, i.e. when transport is catalysed by the PTS or sugar phosphate/phosphate antiport [14–17].

The enzymes of the Leloir pathway of galactose metabolism include galactokinase (GalK) which phosphorylates galactose at the C-1 position, UDPglucose:galactose-1-phosphate uridylyltransferase (GalT) which transfers the UDP group from UDPglucose to galactose-1-phosphate, and UDPglucose 4-epimerase (GalE) which catalyses the epimerization of UDPgalactose to UDPglucose (Fig. 2). UDPglucose synthetase (GalU, not shown) and phosphoglucomutase (Pgm) are also needed for galactose metabolism, but the corresponding genes do not form part of the *gal* operon. This allows expres-

sion of GalU and Pgm when the other Gal enzymes are repressed. In *E. coli* GalU and Pgm are synthesized constitutively [18]. The reversible reactions of the Leloir pathway allow cells to make UDPgalactose (in the absence of exogenous galactose) which can be used for the synthesis of complex (extracellular) polysaccharides.

The majority of 'wild-type' strains of *S. thermophilus* and *L. bulgaricus* cannot metabolize galactose, and in case of *S. thermophilus* the Gal<sup>-</sup> phenotype has been attributed to a defect in the induction mechanism of the galactokinase [19,20]. Under the selective pressure of the chemostat Gal<sup>+</sup> variants of *S. thermophilus* could be isolated, indicating that *galK* is present in this organism [19]. The level of galactokinase was many times greater in the Gal<sup>+</sup> variants than in the parent strain. The failure of wild-type cells to express GalK results in the excretion into the medium of 1 molecule of galactose per molecule of lactose metabolized.

#### Carbohydrate transport ATPases

Recently, a binding protein-dependent transport system facilitating the uptake of multiple sugars, i.e. the galactosides raffinose and melibiose, and isomaltotriose, has been described for *Streptococcus mutans* [21]. The system includes a sugar-binding lipoprotein (MsmE), two membrane proteins (MsmF, MsmG) and a protein (MsmK) containing a highly conserved ATP-binding cassette. The recognition of the ATP-binding cassette is often used to designate the transport systems as members of the ABC transport superfamily [22,23]. The genes encoding the Msm proteins are clustered together with genes encoding a  $\alpha$ -galactosidase (Aga), sucrose phosphorylase (GtfA), dextran glucosidase (DexB), and the Leloir pathway enzymes (Fig. 3). Although the nature of the driving force of the Msm system has not been demonstrated biochemically, the high level of sequence similarity with other ABC transport ATPases makes it likely that uptake is directly energized by ATP [10].

A lactose transport ATPase could also be present in *Leuconostoc (Lc.) lactis*. The amino acid sequences of two *Lc. lactis* polypeptides are ho-

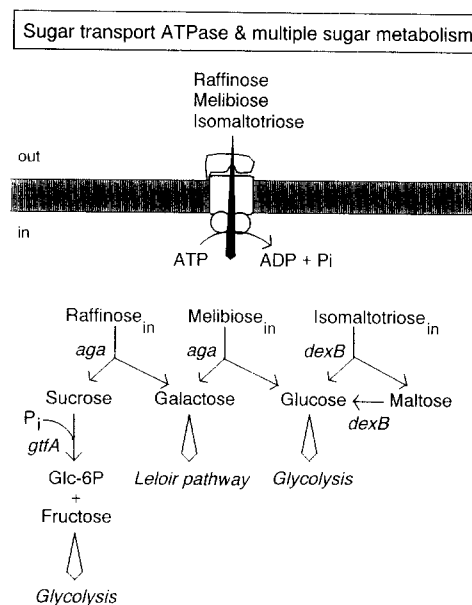


Fig. 3. Sugar transport ATPase and multiple sugar metabolism in *Streptococcus mutans*. *aga*,  $\alpha$ -galactosidase; *dexB*, dextran glucosidase; *gtfA*, sucrose phosphorylase (for details, see Russell et al. [21]).

mologous to the integral membrane proteins of some binding protein-dependent transporters [24]. Surprisingly, expression of these polypeptides in an *Escherichia coli lacY* strain (mutant defective in lactose transport) restores growth of the organism on lactose, while only part of the components of the possible *Lc. lactis* lactose transport ATPase are present.

The number of ATP molecules hydrolysed per solute taken up by the transport ATPases is most likely 1 or 2 [10] which makes transport via these systems energetically expensive as compared to the ion-linked transporters, exchange systems and PTS (see below). Generally the binding protein-dependent transporters bind their substrates with very high affinity, which can be advantageous for an organism since it allows efficient transport in the nano- and submicromolar range of concentrations.

#### Ion-linked sugar transport and sugar exchange mechanisms

Ion-linked galactoside transport has first been described for the atypical *L. lactis* 7962 where methyl- $\beta$ -D-galactopyranoside (TMG) accumula-

tion has been shown to be coupled to the uptake of a proton [25]. The system is highly specific for galactose, TMG and other galactose analogs, but exhibits poor affinity for lactose. A similar system has been described for *L. lactis* ML3 [26]. Lactose transporters driven by the electrochemical proton gradient have been described for *S. thermophilus* and *L. bulgaricus* [27]. These transporters are selective not only for lactose ( $\beta$ -galactoside) but also for melibiose ( $\alpha$ -galactoside), galactose (monosaccharide) and to a lesser extent raffinose (trisaccharide) [28]. Deletion of the chromosomal gene encoding the lactose transport protein (LacS) of *S. thermophilus* abolishes all modes of facilitated galactoside diffusion, demonstrating that a single system catalyses the uptake, efflux and exchange of galactosides (J. Knol, B. Mollet and B. Poolman, unpublished results).

Studies on the kinetic mechanism of the lactose transport protein (LacS) of *S. thermophilus* have indicated that a full translocation cycle involves stoichiometric transmembrane movement of a galactoside and a proton. On the other hand, with saturating concentrations of galactosides (e.g. lactose and galactose) on either side of the membrane, release of substrate (and proton) is immediately followed by (re)binding of substrate (and proton) rather than by reorientation of the 'empty' binding site(s) [29]. Under these conditions the carrier protein performs an exchange reaction with no net proton translocation. Since *S. thermophilus* and *L. bulgaricus* strains excrete galactose into the medium in amounts stoichiometric with the uptake of lactose, the suggestion has been made that under physiological conditions lactose is taken up in exchange for a galactose molecule [27,30]. Evidence in favour of this notion has been obtained from kinetic studies of galactoside transport [29] and comparison of in vivo transport and metabolism rates (Poolman and Foucaud, unpublished). The suggestion that the LacS galactoside transporter of *S. thermophilus* is a strict lactose/galactose antiporter [31] is not correct. Although the lactose/galactose exchange reaction may be favoured under many conditions, the exchange mode simply reflects partial steps, forward and backward reactions with no net proton translocation, of the complete

translocation cycle which involves sugar and proton uptake on one side and release on the other side of the membrane, and reorientation of loaded and unloaded substrate binding sites [29].

The exchange reaction has some distinct advantages over sugar- $H^+$  symport for the uptake of lactose and excretion of galactose. Firstly, although uptake of lactose in symport with  $H^+$  is stimulated by the transmembrane electrochemical proton gradient ( $\Delta p$ ), galactose efflux is inhibited and net exit will only occur if the galactose concentration gradient ( $\Delta\mu_{\text{Gal}}/F$ ) exceeds the  $\Delta p$ . In comparison, the linkage of lactose uptake to galactose excretion via an exchange reaction assures tight coupling to the metabolism, which minimizes (excessive) galactose accumulation and possible product inhibition [30]. Secondly, exchange is less drastically affected by the absolute  $H^+$  concentrations than sugar- $H^+$  symport [29]. The rate of efflux is significantly limited by the release of the proton at the outer surface of the membrane, and, as a consequence, extremely slow below pH 7. Similarly, influx is inhibited by a low intracellular pH. Exchange, on the other hand, proceeds maximally between pH 6–7 and with rates at least one order of magnitude faster than the corresponding sugar- $H^+$  symport reaction.

The genes encoding the lactose (galactoside) transport proteins (LacS) of *S. thermophilus* and *L. bulgaricus* have been cloned, characterized and functionally expressed in *E. coli* [27,28,32,33]. Both proteins are composed of an amino-terminal polytopic hydrophobic membrane domain (carrier domain) and a carboxy-terminal hydrophilic domain. The carrier domain is homologous to the melibiose carrier protein (MelB) of *E. coli*, the xylose carrier protein (XylP) of *Lactobacillus pentosus*, and some other carrier proteins that most likely couple the uphill movement of sugars to the downhill movement of a cation (proton, sodium or lithium) (Fig. 4). The hydrophilic (cytoplasmic) domain of LacS is homologous to various IIA protein(s) or protein domains of PTS, previously indicated as enzyme III or enzyme III-like domain (Fig. 4). The chimeric nature of LacS makes this transporter unique and suggests that it shares features of both ion-linked transporters and PTS.

Carboxy-terminal deletion mutants of LacS have been constructed which lack the entire IIA domain (approximately 170 amino acids; Fig. 4) (B. Poolman and B. Nieuwenhuis, unpublished). Studies with these mutants indicate that the basic properties of the transport reaction are retained in these mutants. In the presence of PEP, purified enzyme I and HPr (the general phosphoryl transfer proteins of the PTS), the IIA domain of the wild-type LacS protein can be phosphorylated at a specific histidine residue (His-552, see Fig. 3) [28]. Currently, the kinetic mechanism of 'phosphorylated' LacS is compared with that of the unphosphorylated protein both in vitro and in vivo.

Galactoside (TMG) transport has also been studied in the heterofermentative lactobacilli *L. brevis* and *L. buchneri* [34]. In the presence of the exogenous energy source arginine, these cells accumulate TMG most likely via a galactoside-H<sup>+</sup>

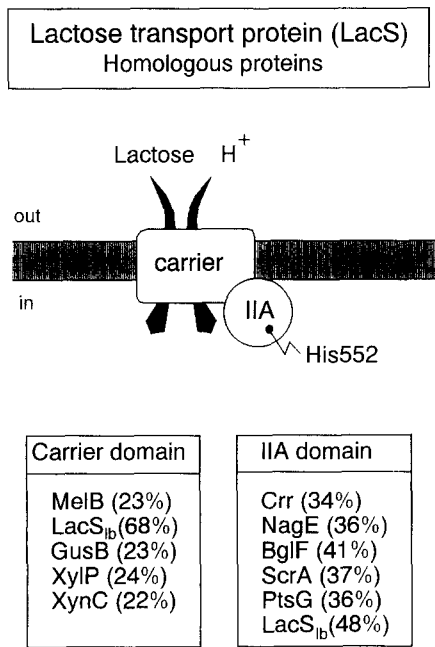


Fig. 4. Model of the lactose transport protein (LacS) of *Streptococcus thermophilus*. Sequence similarity between the carrier domain and other secondary transport proteins, and between the IIA domain and phosphoryl transfer protein(s) domains of the PTS are indicated. His-552 is the PEP/enzyme I/HPr-dependent phosphorylation site (for details, see Poolman et al. [27,28]).

*Gal and lac genes of Streptococcus thermophilus*

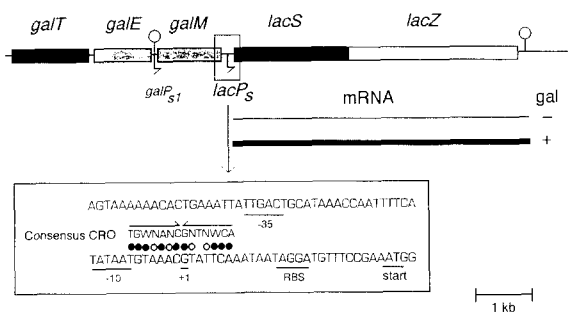


Fig. 5. Organization of the *gal* and *lac* genes in *S. thermophilus*. *galT*, *galE*, *galM*, *lacS* and *lacZ* code for UDPglucose:galactose 1-phosphate uridylyl transferase, UDPglucose 4-epimerase, aldose 1-epimerase (mutarotase), lactose (galactoside) transport protein and  $\beta$ -galactosidase, respectively (see Poolman et al. [27,35]; unpublished results). Promoter and putative transcription termination sequences are depicted. Relative amounts of *lac* mRNA in the presence and absence of galactose are indicated by horizontal bars. CRO refers to a consensus operator sequence of catabolite-repressed promoters in Gram-positive bacteria [38].

symport mechanism. Addition of glucose results in a rapid efflux of TMG, and it has been suggested that glucose converts the ion-linked transport into a uniport mechanism [34]. Since addition of glucose also elicits the phosphorylation of the energy coupling proteins of the PTS, it is conceivable that phosphorylation of a domain similar to IIA in LacS mediates the apparent conversion in transport mechanism.

The lactose transport genes (*lacS*) of *S. thermophilus* and *L. bulgaricus* are organized in an operon that also contains the  $\beta$ -galactosidase gene (*lacZ*) (Fig. 5) [27,35,36]. Downstream of the *lac* operon of *L. bulgaricus* parts of an open reading frame (ORF) have been found. A putative polypeptide encoded by this ORF shows similarity with various repressor proteins, including LacI, GalR, and PurR of *E. coli* [37]. Based on the sequence analysis it has been concluded that this ORF does not represent a functional repressor protein, which is consistent with the constitutive expression of the *lac* genes in *L. bulgaricus*. In contrast, expression of the *lac* genes in *S. thermophilus* is inducible but the regulatory protein(s) have not yet been identified. The genes encoding

aldose 1-epimerase (GalM), UDPglucose 4-epimerase (GalE) and UDPglucose-hexose-1-phosphate uridylyltransferase (GalT) are found upstream of the *lac* operon of *S. thermophilus* (Fig. 5). GalE and GalT form part of the Leloir pathway of galactose metabolism (Fig. 2), but in *S. thermophilus* these enzymes most likely function in the synthesis of precursors (UDPsugars) of complex extracellular polysaccharides. Induction of the *lac* (and *gal*) genes is effected by galactose and galactose analogs, but not by lactose, whereas glucose represses ([35]; Foucaud and Poolman, unpublished). Sequences strongly resembling those found upstream of catabolite-repressed genes of Gram-positive bacteria [38] have been detected in the *lac* promoter region of *S. thermophilus* (consensus CRO, Fig. 5), suggesting that expression of the *lac* genes is also under glucose (catabolite) repression control.

With the exception of the secondary transporters for galactosides described above, there is no further evidence for ion-linked sugar transport or sugar exchange mechanisms in lactic acid bacteria [16]. Recently, the genes responsible for the ability of *L. pentosus* to utilize xylose have been cloned and characterized. In addition to D-xylose isomerase (XylA), D-xylulose kinase (XylB) and a presumed regulatory protein (XylR) [39], an open reading frame (XylP) has been found which displays sequence similarity to the carrier domain of the lactose transport proteins of *S. thermophilus* and *L. bulgaricus*, and a number of other secondary transport proteins (Fig. 4). This system most likely transports xylose in symport with  $H^+$  (J. Martena, M. Posno and B. Poolman, unpublished results).

#### *Sugar phosphate / phosphate antiport*

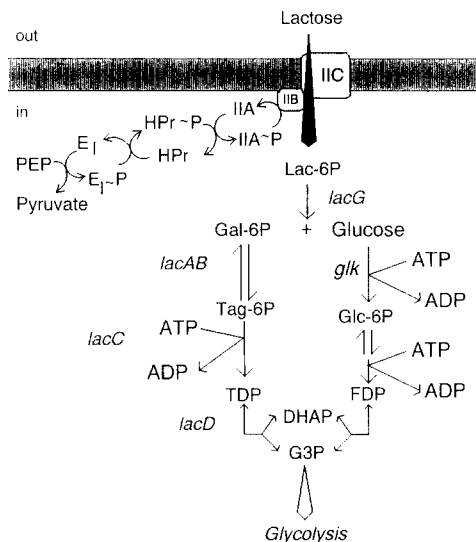
Sugar 6-phosphate/phosphate antiport has been demonstrated first in *L. lactis* [40]. Subsequent studies have shown that this type of anion-exchange is found in various Gram-positive and Gram-negative bacteria. Since anion-exchange is the topic of a number of recent reviews [17,41], only the basic properties of the antiporter mechanism will be discussed. The features of the sugar 6-phosphate/phosphate antiporter of *L. lactis* are: (i) the system catalyses homologous and het-

erologous exchange of phosphate and sugar 6-phosphates; (ii) substrate specificity studies have shown that arsenate can replace phosphate, and that the apparent affinity constants ( $K^{App_i}$  or  $K^{App_o}$ ) for the organic sugar phosphates are in the order: 2-deoxyglucose 6-phosphate, mannose 6-phosphate, glucose 6-phosphate < fructose 6-phosphate < glucosamine 6-phosphate < ribose 5-phosphate < others; (iii) the  $V_{max}$  of homologous phosphate exchange is approximately 5-fold faster than the  $V_{max}$  of heterologous exchange; (iv) the transport system favours monovalent phosphate but takes randomly the available mono- and divalent sugar 6-phosphates; (v) the exchange is electroneutral under all conditions; (vi) to maintain electroneutrality during heterologous exchange, the antiport system catalyses phosphate/sugar 6-phosphate exchange with a pH-dependent variable stoichiometry. At pH 7.0 (0.9 pH units above the  $pK_2$  of glucose 6-phosphate) the carrier catalyses exchange of two molecules of monovalent phosphate for one molecule of divalent glucose 6-phosphate, whereas at pH 5.2 (0.9 pH units below the  $pK_2$  of glucose 6-phosphate) the antiporter takes one molecule of monovalent phosphate for one molecule of monovalent glucose 6-phosphate.

The significance of catalysing sugar 6-phosphate/phosphate exchange is not quite understood since sugar phosphates are usually not present in the bacterial environment. At the same time, cells need to prevent leakage of these energy-rich intermediates. For *E. coli* and *Salmonella typhimurium* sugar phosphate uptake coupled to phosphate excretion could have some relevance in habitats like the intestinal tract of warm-blooded animals, in which phosphorylated sugar intermediates can be found [42]. In lactic acid bacteria, the reverse reaction, i.e. phosphate uptake at the expense of sugar phosphate excretion, has been implicated as a defence mechanism against unregulated sugar phosphate production, which can be bacteriocidal [43].

A system catalysing phosphate self-exchange has been identified in *Streptococcus pyogenes* [44]. At present, no physiological role can be assigned to this transport system since only phosphate and arsenate can serve as substrates.





*PEP:sugar phosphotransferease systems*

lactose PTS of *Lb. casei*, evidence has been obtained that a cysteine (Cys-384), rather than a histidine residue, mediates the final phosphoryl transfer to lactose [47].

In addition to catalysing galactoside uptake concomitant with phosphorylation of the sugar, IIBC of the lactose PTS is also believed to catalyse efflux (expulsion) of intracellularly formed free galactosides [14,46]. This expulsion of galactosides is phenomenologically similar to that observed in the heterofermentative lactobacilli where a secondary transport mechanism has been implicated in the efflux of free galactosides (see above). For details on galactoside efflux in lactic acid (and other Gram-positive) bacteria the reader is referred to Reizer [48].

The genes encoding IIBC (or lactose specific enzyme II) and IIA (previously, factor III<sup>Lac</sup> or enzyme III-lac) components of the lactose PTS and those coding for the phospho- $\beta$ -galactosidase of *L. lactis* and *L. casei* have been cloned and sequenced [47,49–52]. The genes are organized in an operon structure which has best been studied for *L. lactis*. The lactose operon of *L. lactis* also comprises the tagatose 6-phosphate pathway genes [53] (Fig. 6). The *lac* genes of *L. lactis* and *Lb. casei* are induced during growth on lactose and galactose. A repressor protein (LacR) acting at the level of transcription mediates the expression of the *lac* genes in *L. lactis* [53–55]. The interaction of LacR with the *lac* operator region is inhibited by tagatose 6-phosphate, and this phosphorylated intermediate rather than galactose 6-phosphate most likely functions as inducer of the *L. lactis lac* operon [56]. LacR belongs to the family of DNA-binding proteins that also includes LacR of *Staphylococcus aureus* and the *E. coli* repressors DeoR, FucR and GutR [54].

When *lacR* of *L. lactis* is deleted, the lactose PTS and phospho- $\beta$ -galactosidase activities of cells grown on glucose are approximately 50% of those grown in the presence of lactose [57]. This suggests that expression of the *lac* operon is not only effected by the LacR repressor but is also subject to glucose (catabolite) repression. A region homologous to a sequence involved in catabolite repression in bacilli is found downstream of one of the operator elements of the *L.*

Table 1

Sugar transport and metabolism in *Streptococcus thermophilus*

Substrate	Growth	Transport mechanism	
		PEP-PTS	Secondary transport
Lactose	+	—	+
Galactose	—	—	+
Glucose	+ / —	—	?
Sucrose	+	+	—
Fructose	+	+	—

*lactis* (and *Staphylococcus aureus*) *lac* operon. As noted above a similar region is also present in the promoter region of the 'non-PTS' *lac* operon of *S. thermophilus* ('CRO' in Fig. 5).

The main transport system for glucose in *L. lactis* is a PTS that is often referred to as mannose PTS or glucose/mannose PTS since the sugar specificity resembles that of the mannose PTS of *E. coli*, i.e. relatively high affinity for glucose, 2-deoxy-D-glucose (2DG) and mannose, while  $\alpha$ -methylglucose is not a substrate [58,59]. Mannose PTS defective mutants of *L. lactis* ML3 and 133 have been isolated on basis of resistance to 2DG [43]. These mutants still grow on glucose. However, mutants defective in both the mannose PTS and hexokinase fail to grow in media containing glucose, suggesting that another non-PTS transport system for glucose is present in the lactococci [60].

The presence of a glucose PTS and/or a mannose PTS has been suggested for a large number of lactic acid bacteria although often the evidence is indirect [16]. In most of these bacteria glucose is the preferred substrate and its presence inhibits the utilization of other sugars. In *S. thermophilus* and *L. bulgaricus*, on the other hand, glucose is a poor substrate relative to the disaccharides lactose and sucrose. With glucose as carbon and energy source, the lag time preceding exponential growth is long and the growth rates are lower than with lactose or sucrose. In a study on the sugar transport mechanisms in *S. thermophilus* (strain A147), PTS activity could be detected with sucrose and fructose as substrate but not with glucose, lactose and galactose (Table 1) (C. Foucaud and B. Poolman, unpublished results). Lactose and galactose are transported by

the LacS secondary carrier protein, but a similar mechanism could not be detected for glucose.

### Precursor / product exchange and deiminase pathways

Various lactic acid bacteria have the ability to metabolize arginine via the arginine deiminase (ADI) pathway [6,61]. The ADI pathway includes (i) arginine deiminase, which catalyses the conversion of arginine into citrulline plus ammonia in an essentially irreversible reaction; (ii) ornithine carbamoyltransferase, which catalyses the phosphorolysis of citrulline, yielding ornithine plus carbamoylphosphate (this step is thermodynamically limiting since the equilibrium of this reaction strongly favours the formation of citrulline); (iii) carbamate kinase, which catalyses the reversible conversion of carbamoylphosphate plus ADP into ATP, carbon dioxide plus ammonia; (iv) an antiporter, which catalyses a one-to-one exchange of arginine (substrate, precursor)

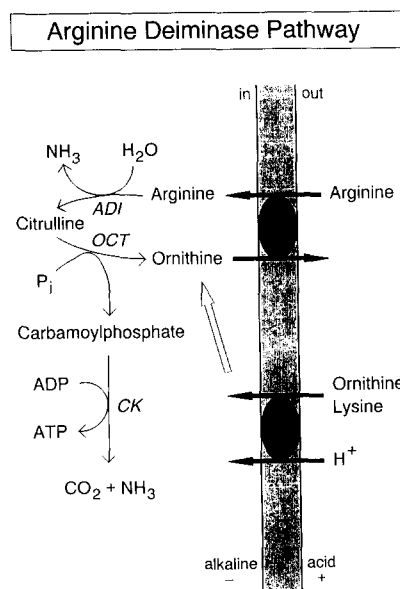


Fig. 7. Arginine/ornithine antiport and the arginine deiminase pathway. Accumulation of ornithine (lysine) via the the  $\Delta p$ -driven lysine transport system is also depicted in the figure. ADI, arginine deiminase; OCT, ornithine carbamoyltransferase; CK, carbamate kinase.

for ornithine (product) [5,6] (Fig. 7). Overall, the ADI pathway yields 1 mol of ornithine and carbon dioxide, 2 mol of ammonia, and 1 mol of ATP by substrate level phosphorylation per mol of arginine metabolized [6,61]. A similar deiminase pathway is used for the conversion of agmatine into putrescine in *Enterococcus faecalis*, in which case an analogous transport system for cationic metabolites is associated with the agmatine deiminase (AgmDI) pathway [8]. Since the driving force for the uptake of these cationic substrates (precursors) and excretion of the products is supplied by the precursor and product concentration gradients, no additional metabolic energy is required for the translocation of these solutes. Consequently, the net metabolic energy yield of arginine and agmatine metabolism by the deiminase pathways is 1 mol of ATP per mol of substrate metabolized.

Detailed kinetic analysis of arginine/ornithine antiport indicates that the carrier possesses a single substrate-binding site which is present alternately at the inner and outer surface of the cytoplasmic membrane [62]. The exchange reaction catalysed by the antiporter resembles a 'ping-pong' mechanism regarding enzyme kinetics. Thus, arginine (substrate) associates at the outer surface of the membrane, the carrier-substrate complex reorients its binding site and arginine dissociates at the inner surface. Rapid metabolism of arginine assures that the intracellular arginine concentrations are kept low compared to those of ornithine [6]. Next, ornithine (product) binds, the carrier-product complex reorients its binding site and ornithine is released into the medium. Under conditions that a large fraction of arginine is converted into ornithine, arginine metabolism will slow down because ornithine competes with binding of arginine at the outer surface of the membrane.

Since uptake of arginine and excretion of ornithine via the antiporter protein are tightly linked, questions arise with respect to the initiation of arginine metabolism and replenishment of the ornithine pool when a fraction of the arginine is used for biosynthetic purposes. *L. lactis* has solved this dilemma by taking advantage of a  $\Delta p$ -driven lysine transport system that can accept

ornithine with low affinity [63]. Furthermore, the antiporter catalyses heterologous exchange of arginine for lysine in addition to exchange of arginine for ornithine (Fig. 7). Accumulation of lysine via the  $\Delta p$ -driven transport system in combination with exchange of lysine for arginine via the antiporter results in cyclic transport of lysine and net accumulation of arginine at the expense of the  $\Delta p$ . In this scheme a high level of ornithine can be sustained when part of the arginine is used for biosynthesis.

### Precursor/product exchange and decarboxylation reactions

Malolactic fermentation is carried out by some species of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* [64]. In this pathway L-malate enters the cells and is decarboxylated by malolactic enzyme to yield L-lactic acid and carbon dioxide, after which the products leave the cell. Although the decarboxylation reaction does not yield metabolic energy directly, the free energy of the decarboxylation reaction can be conserved via an indirect  $H^+$  pump mechanism. It has been shown for *L. lactis* that L-malate utilization results in the formation of a  $\Delta p$  that is sufficiently high to drive ATP synthesis via the  $F_0F_1$ -ATPase [9].

The transport of L-malate has been characterized in artificial membranes prepared from *L. lactis* IL1403 and in isogenic mutants, that are defective in either L-malate transport or L-malate decarboxylation [9]. These studies have shown that monoanionic L-malate ( $MH^-$ ) is taken up either in exchange for L-lactic acid (LH) or as  $MH^-$  uniport (Fig. 8). In both cases a negative charge is translocated from the medium to the cytoplasm which results in the formation of a  $\Delta\psi$  (inside negative). Since charge compensation in the decarboxylation reaction requires the consumption of a proton, the cytoplasm is alkalinized relative to the outside medium and a  $\Delta pH$  is formed. Thus, the free energy of the decarboxylation reaction is converted into a  $\Delta p$  ( $=\Delta\psi - Z\Delta pH$ ) simply by compartmentalization of the malolactic fermentation pathway, i.e. stoichio-

metric uptake of L-malate and excretion of L-lactic acid into the medium combined with decarboxylation in the cytoplasm.

A similar mechanism of metabolic energy conservation has recently been discovered in *Lactobacillus buchneri*. This organism decarboxylates histidine to the biogenic amine histamine, which is excreted into the medium [11]. The  $pK_a$ s of the imidazole ring of histidine and histamine are 6.0 and 5.8, respectively, and the protonation state of the side chain of both molecules will have a similar dependency on pH. However, histamine carries one additional positive charge which makes the histidine/histamine exchange electrogenic, resulting in the net movement of a positive charge to the outside (Fig. 9).

Biogenic amines like cadaverine, putrescine, tyramine and tryptamine, frequently found in food products, can be formed by decarboxylation of the corresponding amino acids [65–67], and mechanisms similar to malate/lactic acid and histidine/histamine exchange may form part of

#### Electrogenic histidine/histamine exchange and histidine decarboxylation

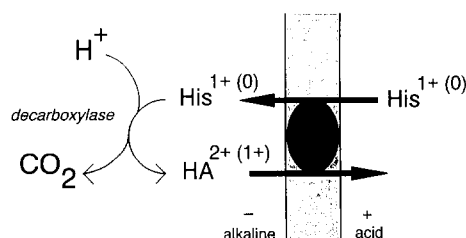


Fig. 9. Schematic representation of metabolic energy conservation by histidine/histamine exchange and histidine decarboxylation.

the decarboxylation pathways and contribute to metabolic energy conservation. It is worthwhile recalling that putrescine can be formed from the decarboxylation of ornithine but also from the conversion of agmatine via the agmatine deiminase pathway. As described above for the agmatine deiminase pathway of *Enterococcus faecalis*, agmatine and putrescine are transported via an electroneutral antiport [8]. Evidence for putrescine/ornithine exchange as part of the ornithine decarboxylation pathway of *E. coli* has been presented [68]. Unfortunately, the expected electrogenicity of ornithine/putrescine exchange has not been addressed adequately. Finally, there is some evidence that *Lactobacillus* sp. are able to convert aspartate into alanine and gain metabolic energy from this reaction most likely as a result of aspartate/alanine exchange in combination with the decarboxylation (K. Abe, unpublished results).

The energetic consequences of electrogenic transport in combination with precursor decarboxylation can be calculated from the standard free energy ( $\Delta G'_0$ ) of the decarboxylation reaction,  $pCO_2$  values and assuming that the reaction proceeds to equilibrium [11]. For malolactic fermentation at pH 7, a  $pCO_2$  of 1 bar and about equal concentrations of dianionic malate and lactate, a  $\Delta p$  of  $-275$  mV is thermodynamically feasible (The  $\Delta G'_0$  of the malate decarboxylation reaction at pH 7.0 and  $CO_2$  at 1 bar is approximately  $-26.5$  kJ mol $^{-1}$  [69]; for the lysine to

#### Energy coupling by malolactic fermentation

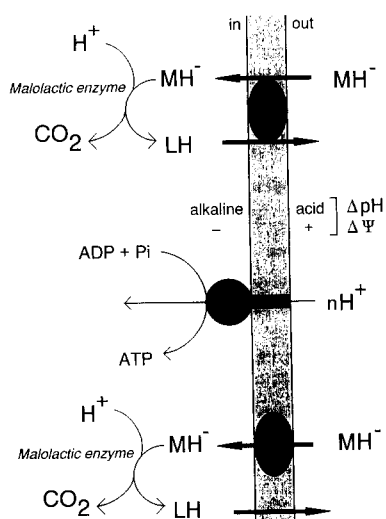


Fig. 8. Schematic representation of metabolic energy conservation by malolactic fermentation.  $MH^-$ , mono-anionic malate; LH, lactic acid. Malate uptake is shown as  $MH^-/LH$  exchange and  $MH^-$  uniport [9]. Exit of lactic acid can occur via the exchange reaction or by passive and/or carrier-mediated diffusion.

cadaverine conversion the  $\Delta G'_0$  is approximately  $-25 \text{ kJ mol}^{-1}$  [11]. Notice that the  $\Delta G$  of this type of reactions becomes more negative when the pH decreases.). Since the precursor and product of the decarboxylation reactions are structurally related, the product will competitively inhibit the binding and transport of the precursor. Furthermore, at some point the  $\Delta \text{pH}$  generated results in an increased intracellular pH that is likely to slow down the decarboxylation reaction. Thus for kinetic reasons the  $\Delta p$  formed will be less than predicted on basis of thermodynamic equilibrium.

In case of malate decarboxylation (malolactic fermentation) in *L. lactis* and histidine decarboxylation in *L. buchneri*, maximal  $\Delta p$  values of  $-175$  and  $-145 \text{ mV}$ , respectively, have been measured [9,11]. Given a stoichiometry of 3  $\text{H}^+$ /ATP for ATP synthesis by the  $\text{F}_0\text{F}_1$ -ATPase [147], a  $\Delta p$  of  $-175$  can yield (at thermodynamic equilibrium) a phosphorylation potential ( $\Delta G'_p/F$ , expressed in electrical units (mV)) of  $-525 \text{ mV}$ . Notice that  $\Delta G'_p/F$  values for resting and growing cells *L. lactis* are about  $-360$  and  $-460 \text{ mV}$ , respectively [70]. By comparing the  $\Delta G'_0$  values (and also the  $\Delta G$ , at physiologically relevant concentrations of the reactants) of the decarboxylation reactions with the  $\Delta G'_p$  values it becomes clear that thermodynamically the changes in free energy of these reactions are too small to be conserved in the form of ATP (directly). However, the combined action of an electrogenic transport together with the decarboxylation allows a significant portion of the free energy to be conserved in ion-gradients.

### ATP-driven nutrient uptake and product/drug excretion

The variety of nutrient uptake systems in lactic acid bacteria has extensively been dealt with in a previous review [16]. At that time a number of systems were categorized as phosphate-bond driven transport proteins, i.e. the glutamate/glutamine, asparagine and phosphate transport systems of *L. lactis*, and the transport systems for acidic amino acids and phosphate in *E. hirae*

(and *S. mutans*) (Table 2). Although not all of these systems have been studied to the same extent, several common properties have been recognized:

1. Transport proceeds in the absence of a  $\Delta p$ .
2. The concentration gradients of the solutes (in/out) can be up to  $10^5$  which exceeds the thermodynamic limits set by the  $\Delta p$  (assuming that one  $\text{H}^+$  accompanies the translocation of one solute molecule).
3. Metabolic inhibitor studies, and apparent relationships between transport activities and intracellular ATP concentrations, suggest that ATP or an equivalent energy-rich phosphorylated intermediate supplies the metabolic energy for the translocation process.
4. Transport is regulated by the intracellular pH in most cases.
5. Transport is unidirectional, i.e. the rates of uptake are at least two orders of magnitude higher than the rates of exit.
6. At least some of the systems are inhibited by the presence of substrate on the inner surface of the membrane (*trans* inhibition).

More recently, a constitutively expressed high-affinity uptake system for glycine betaine, an osmoregulated low-affinity proline uptake system and the oligopeptide transport protein of *L. lactis* have been added to the list of lactococcal phosphate-bond driven transport systems [71,72]. Also transport systems for glutamate, glutamine/asparagine, and, biotine, folate and thiamine in *L. casei* are unaffected by the magnitude of  $\Delta p$ , and are most likely driven by phosphate-bond energy [73,74]. The binding protein-dependent multiple sugar transport system (MsmEFGK) of *S. mutans* has been described above.

Of the phosphate-bond energy-dependent transport systems only the genes encoding the *S. mutans* MsmEFGK system [21] and the *L. lactis* oligopeptide transporter have been isolated and characterized [75]. The oligopeptide transport system is composed of five proteins, encoded by the *oppFDCBA* operon. OppD and OppF are homologous to the ATP binding protein(s) (domains) of the ABC-transporter superfamily [22,23], and most likely form the target for the (complete) inhibition of oligopeptide transport by

*ortho*-vanadate [72]. OppB and OppC are highly hydrophobic proteins, that on basis of hydropathy analyses, are able to span the cytoplasmic membrane in  $\alpha$ -helical configuration six times. OppB and OppC are homologous to the corresponding polypeptides of the oligopeptide transporters of *S. typhimurium* and *B. subtilis*, and DciAB and DciAC of the dipeptide transport system of *Bacillus subtilis*. The transmembrane domains of OppB and OppC would constitute the pathway that facilitates the translocation of the oligopeptides across the cytoplasmic membrane. OppA is homologous to the peptide binding proteins of *S. typhimurium* and *B. subtilis*.

Since Gram-positive bacteria do not have a periplasmic space, the question whether these bacteria possess binding protein-dependent transport systems analogous to those found in Gram-negative bacteria could only be addressed after the primary sequences of the proteins became available. It has turned out that Gram-positive species have the equivalent systems with binding proteins that are anchored to the membrane by a lipid group, which restricts diffusion to two dimensions (in the plane of the membrane) [76,77]. The sugar-binding protein MsmE of *S. mutans* has been shown to be attached to the membrane by means of an amino-terminal lipo-amino acid anchor (Figs. 1 and 3; [78]). Known prokaryotic lipoproteins have a glyceride linked to a cysteine residue, and a consensus sequence (Leu-Ala-Ala/Gly-↓Cys) around the the cysteine in the prolipoprotein has been proposed [79]. OppA of *L. lactis* possesses the consensus prolipoprotein cleavage site Leu-Ser-Ala-↓Cys between residues 22–23 [75].

The functional characteristics of the lactococcal Opp system strongly resemble those of the other phosphate-bond-dependent transport systems (Table 2), of which the glutamate/glutamine transporter of *L. lactis* has been studied in greatest detail [80–82]. It is conceivable that these systems have a structural complexity similar to Opp, and that a substrate-binding lipoprotein serves as the initial receptor for transport by delivering the substrates to the integral membrane components. Only in a few cases ATP hydrolysis has been demonstrated rigorously to

energize binding protein-dependent transport [83,84]. The mechanism by which hydrolysis of ATP is coupled to the solute translocation process is still not clear (for recent reviews, see [23,85]). The energy requirements of phosphate-bond-dependent transport in lactic acid bacteria (and other Gram-positive species) indicate that ATP plays a role in the energization of transport, but unequivocal evidence is still lacking. Finally, it is worth emphasizing that although these ATP-driven transporters facilitate transport in the absence of a  $\Delta p$ , the systems are highly sensitive to changes in the intracellular pH [80,86,87]. Consequently, changes in the  $\Delta pH$  component of  $\Delta p$  are directly reflected in changes in transport activity and can easily lead to misinterpretations regarding the mechanism of energy coupling to these systems [87].

The ATP-dependent transport systems described so far only catalyse nutrient uptake and exhibit little or no efflux activity under physiological conditions. In a study on the regulation of the intracellular pH in *L. lactis*, employing the fluorescent probe 2',7'-bis-(2-carboxyethyl)-5( and 6)-carboxyfluorescein (BCECF), energy-dependent efflux of BCECF has been observed [88]. A further characterization of this efflux process indicated that most likely ATP serves as energy donor [89]. In search of the physiological function of this BCECF efflux pump, and owing to its functional resemblance to eukaryotic P-glycoprotein (Mdr1), a variety of compounds/drugs (antibiotics, Mdr1 substrates and inhibitors, uncouplers, ionophores etc.) have been tested as possible substrate of the BCECF efflux pump. None of these toxic compounds could serve as substrate for the BCECF efflux pump [89]. To some of the compounds tested (e.g. ethidium-bromide), however, *L. lactis* mutants with increased resistance could be isolated [90]. These mutants confer cross-resistance towards various toxic compounds such as the Mdr1 substrates daunomycin and actinomycin D, the *Plasmodium falciparum* multidrug resistance (mdr) substrate chloroquine, the bacterial mdr (Bmr) substrate rhodamine 6-G, and ionophores like nigericin (for a review on bacterial excretion systems see Poolman [10]). Resistance of the cells to these molecules is conferred by a transport

Table 2

Transport mechanisms in lactic acid bacteria

Substrate specificity	Organisms	Proteins
<b>H<sup>+</sup>-Symport</b>		
Leucine, isoleucine, valine	<i>L. lactis</i> , <i>L. plantarum</i> , <i>Lc. mesenteroides</i> , <i>L. casei</i>	–
Alanine, glycine	<i>L. lactis</i> , ( <i>S. thermophilus</i> )	AlaT <sup>a</sup>
Serine, threonine	<i>L. lactis</i>	–
Alanine, glycine, serine, threonine	<i>E. hirae</i> , <i>S. pyogenes</i>	–
Lysine, ornithine	<i>L. lactis</i> , ( <i>L. plantarum</i> )	–
Phenylalanine, tyrosine, tryptophane	( <i>L. lactis</i> , <i>L. casei</i> )	–
Di- and tripeptides	<i>L. lactis</i>	DtpT
Citrate	<i>Lc. mesenteroides</i> , <i>L. lactis</i> , <i>Lc. lactis</i>	CitP
Lactose, galactose, melibiose, TMG, raffinose	<i>S. thermophilus</i> <sup>b</sup> , <i>L. bulgaricus</i> <sup>b</sup>	LacS
Galactose	<i>L. lactis</i>	–
TMG	<i>L. lactis</i> , ( <i>L. brevis</i> , <i>L. buchneri</i> , <i>L. plantarum</i> )	–
(Xylose)	<i>L. pentosus</i>	XylP
<b>Na<sup>+</sup>-Symport</b>		
Alanine, (glycine)	<i>S. bovis</i>	–
Serine, threonine	<i>S. bovis</i>	–
<b>H<sup>+</sup>-Antiport</b>		
Calcium	<i>L. lactis</i>	–
Sodium	<i>E. hirae</i>	–
<b>Substrate/product antiport (exchange)</b>		
Arginine/ornithine	<i>L. lactis</i> , <i>E. faecalis</i> , <i>S. sanguis</i> , <i>S. milleri</i>	–
Agmatine/putrescine	<i>E. faecalis</i>	–
Sugar-phosphate/phosphate	<i>L. lactis</i>	–
Malate/lactic acid	<i>L. lactis</i> <sup>c</sup>	–
Histidine/histamine	<i>L. buchneri</i>	–
(Tyrosine/tyramine)	<i>L. buchneri</i>	–
(Aspartate/alanine)	<i>Lactobacillus</i> sp.	–
Phosphate/phosphate	<i>S. pyogenes</i>	–
<b>ATP-driven uptake</b>		
Glutamate, glutamine	<i>L. lactis</i>	–
Glutamate, aspartate	<i>E. hirae</i> , <i>S. mutans</i> , <i>L. casei</i>	–
Asparagine	<i>L. lactis</i> , ( <i>L. casei</i> )	–
Proline, glycine betaine	<i>L. lactis</i>	–
Phosphate	<i>L. lactis</i> , <i>E. hirae</i>	–
Oligopeptides	<i>L. lactis</i>	OppFDCBA
Biotine, folate, thiamine	<i>L. casei</i>	–
Potassium	<i>E. hirae</i>	Ktrl
Raffinose, melibiose, isomaltotriose	<i>S. sanguis</i>	MsmEFGK
<b>ATP-driven efflux</b>		
Proton	<i>E. hirae</i> , (various other lactic bacteria)	F <sub>0</sub> F <sub>1</sub>
BCECF	<i>L. lactis</i>	–
Calcium	<i>E. hirae</i> , <i>S. sanguis</i> , <i>L. lactis</i>	–
(Lactococcin A)	<i>L. lactis</i>	LcnCD
(Nisin)	<i>L. lactis</i>	NisT
(Pediocin PA-1)	<i>P. acidilactici</i>	PedD
<b>ATP-driven exchange</b>		
Sodium/potassium	<i>E. hirae</i>	Ktrl

system that removes various hydrophobic peptide compounds, drugs, and other (toxic) molecules from the cells. Apparently, lactic acid bacteria possess a number of transport systems that are involved in the excretion of compounds that are normally not encountered by the cells, but provide the cells with mechanisms to clean up the cytoplasmic environment when needed.

The driving force for secretion by the multiple drug resistance pump of *L. lactis* has not yet been established, but some of its properties are similar to that of eukaryotic Mdr1. Mdr1 belongs to the family of ABC transporters by the fact that it has the highly conserved ATP-binding cassette in two separate domains as part of a single polypeptide that also contains the transmembrane domains, the two-times-six  $\alpha$ -helical segments [23]. Partially purified Mdr1 catalyses transport in response to ATP hydrolysis [91], and, similar to the BCECF efflux pump and the oligopeptide transporter of *L. lactis* [72,89], the eukaryotic multidrug efflux pump is inhibited by *ortho*-vanadate [92].

Recently, an *E. coli* strain that is highly sensitive to ethidium has been used to select for resistance towards high concentrations of this compound upon complementation with a chromosomal library of *L. lactis* DNA (H. Bolhuis, D. Molenaar, B. Poolman, and W.N. Konings, unpublished results). The putative *L. lactis* mdr gene(s) (*lmr*) are present on a DNA fragment of less than 3 kb. The *lmr* gene(s) and their functional expression in *E. coli* are characterized, and insertion inactivation of the chromosomal gene(s) in *L. lactis* should establish whether the cloned gene(s) correspond with those that upon mutation confer a high cross-resistance towards various toxic compounds to *L. lactis*.

Molecular analysis of the lactococcin A gene

cluster from *L. lactis* subsp. *lactis* biovar *diacetyl-lactis* has revealed the presence of two open reading frames, designated LcnC and LcnD, which are homologous to *E. coli* HlyB and HlyD, respectively [93]. The HlyB protein exports hemolysin across the cytoplasmic membrane, whereas HlyD, which is co-expressed with HlyB, is likely to participate in hemolysin transport across the outer membrane of *E. coli* [94]. Even though HlyB possesses the ATP-binding cassette typical of the ABC superfamily, hemolysin export by HlyB seems to require a  $\Delta p$  (in addition to ATP) [95]. Despite some uncertainties in the mechanism of energy coupling to these excretion processes, and the precise role of the individual components (e.g. HlyD in *E. coli*; role of LcnD in *L. lactis* which does not have an outer membrane), it is fair to say that LcnC and LcnD are likely to constitute a secretion pathway for lactococcin(s) in *L. lactis* that is dependent on ATP [96]. A similar secretion machinery composed of proteins homologous to HlyB (LcnB) and HlyD (LcnD) has been proposed for the translocation across the membrane of the antimicrobial peptide subtilin (lantibiotic) of *B. subtilis* [97]. Also for transport across the cytoplasmic membrane of the lantibiotic nisin from *L. lactis* [98] and the bacteriocin PA-1 from *Pediococcus acidilactici* [99], proteins homologous to the HlyB family are likely to play a role.

Secretion of hemolysin A by HlyB/HlyD is signal sequence independent as opposed to protein translocation by the Sec machinery. By analogy with hemolysin A secretion, translocation of polypeptides across the cytoplasmic membrane by proteins homologous to HlyB/HlyD is often designated 'signal sequence independent'. It needs to be stressed that bacteriocins and lantibiotics from lactic acid bacteria are synthesized as pre-

Note to Table 2:

<sup>a</sup> Alanine transport mutants have been isolated on basis of resistance to  $\beta$ -chloro-alanine [72].

<sup>b</sup> LacS protein that catalyses galactoside-H<sup>+</sup> symport and (heterologous) sugar exchange; lactose/galactose exchange most likely dominates during lactose metabolism (see text).

<sup>c</sup> System also catalyses monoanionic L-malate uniport (see Fig. 8).

Brackets indicate preliminary evidence for the transport mechanism in the particular organism. The genes encoding DtpT, CitP, LacS, XylP, OppDFBCA, MsmEFGK, F<sub>0</sub>F<sub>1</sub>-ATPase subunits, LcnCD, NisT and PedD have been isolated and sequenced (see text). Transport of sugar by the PTS is not indicated.



cursors, and that the corresponding presequences could play a role in the translocation of the precursors across the cytoplasmic membrane. Since only the mature bacteriocins and lantibiotics have antimicrobial activity, the presequence could also serve as a means to protect the producing strain [96].

### ATP-driven cation transport

The major ATPase of lactic acid bacteria is the N,N'-dicyclohexylcarbodiimide (DCCD)-sensitive  $F_0F_1$ -ATPase which pumps protons at the expense of ATP but can also function as ATP synthetase [100]. ATP synthesis by the  $F_0F_1$ -ATPase occurs when  $n \times \Delta p > \Delta G'_p$ , in which  $n$  represents the number of protons translocated ( $n$  is most likely 3),  $\Delta p$  is the proton motive force and  $\Delta G'_p$  is the phosphorylation potential. Thus when a high  $\Delta p$  is generated by osmotic reactions, e.g. product efflux, solute decarboxylation reactions (see above), the metabolic energy in the form of an electrochemical  $H^+$  gradient can be used to drive ATP synthesis. Under conditions that ATP is formed by substrate level phosphorylation, e.g. glycolysis, deiminase pathways, the  $F_0F_1$ -ATPase will function as a hydrolase in order to generate a  $\Delta p$ .

Besides serving as a converter of metabolic energy ( $ATP \rightleftharpoons \Delta p$ ), the  $F_0F_1$ -ATPase of lactic acid bacteria plays a major role in regulating the intracellular pH as has been shown convincingly for *E. hirae* [101–105]. In fact, it has been shown that *E. hirae* can grow under conditions that  $\Delta p$  is zero, provided the medium is slightly alkaline and contains high concentrations of  $K^+$  and nutrients, while  $Na^+$  concentrations are kept low [106]. This further demonstrates that regulation of the intracellular pH (by the  $F_0F_1$ -ATPase and perhaps other ion-translocating enzymes) is crucial for the survival of (lactic acid) bacteria [87]. The role of the  $\Delta p$  in (lactic acid) bacteria is indicated by the severe inhibition of growth in the absence of a  $\Delta p$  when the concentrations of nutrients are in the sub-millimolar rather than in the millimolar range or when high concentrations of  $Na^+$  are present [106]. Thus rapid growth of

(lactic acid) bacteria requires the accumulation of various essential nutrients and excretion of unwanted products for which the  $\Delta p$  supplies the driving force. Various kinds of information are available on the function, structure and regulation of  $F_0F_1$ -ATPases in (lactic acid) bacteria [102,104,105,107–109], but these aspects are not included in this review.

Transport of various cations in lactic acid bacteria is dependent on ATP or a related phosphate bond intermediate. These systems will only be summarized here (see also Table 2, reviewed in Konings et al. [16], and include vanadate-sensitive  $Ca^{2+}$  efflux systems in *L. lactis*, *E. hirae* and *Streptococcus sanguis* [110,111], a vanadate-sensitive  $K^+$ -ATPase in *E. hirae* (see also below; [112–114], a constitutive  $K^+$  transport system (KtrI) in *E. hirae* [115] and a  $Na^+$ -ATPase in *E. hirae* (KtrII) that expels  $Na^+$  in exchange for  $K^+$  [116–118]. The KtrI  $K^+$  uptake system requires ATP in addition to a  $\Delta p$  for transport, and has been claimed to co-transport  $K^+$  and  $H^+$  [115]. KtrI has properties that coincide with the  $K^+$ -ATPase of *E. hirae*, which originally has been proposed to function as a  $\Delta p$ -regulated, electrogenic ATP-driven  $K^+$  uptake system [113,114]. More recently, this ATPase has been suggested to be a  $H^+$  pump [119] and the observed  $K^+$  transport has been attributed to secondary movements of  $K^+$  via leak pathways [118]. KtrII is induced by  $Na^+$  and although the enzyme catalyses a similar exchange as the ouabain-sensitive  $Na^+K^+$ -ATPase of mammalian cells [116,120], the mechanism of ion coupling is quite different [118]. Unlike the  $Na^+/K^+$ -ATPase of mammalian cells the *E. hirae* enzyme is not a P-type ATPase and is not inhibited by *ortho*-vanadate.

### Ion-linked nutrient uptake

Next to *E. coli*, solute transport in bacteria as a whole has perhaps best been studied in *L. lactis*, and so far all cation-linked transport systems in this lactic acid bacterium that have been studied use  $H^+$  as coupling ion. Evidence for  $Na^+$ -coupled transport comes from studies in *Streptococcus bovis* [121].

Under section 'ATP-driven nutrient uptake and product/drug excretion' it has been shown that amino acids with acidic (Glu, Asp) and amide side chains (Gln, Asn) as well as proline are transported by ATP-driven mechanisms in all lactic acid bacteria tested, i.e. *L. lactis*, *E. hirae*, *S. mutans* and *L. casei* (Table 2). Although these amino acids together with glycine betaine, potassium ions, and some other compounds do play a role in the mechanism that confers resistance to osmotic stress [71,122], it is not immediately clear why these solutes in particular are taken up by ATP-driven mechanisms whereas (most) other amino acids are transported by solute- $H^+$  symport (Table 2). The branched chain aliphatic amino acids (Leu, Ile, Val), the neutral amino acids (Ala, Gly), the aliphatic amino acids with a hydroxyl side chain (Ser, Thr) and the aromatic amino acids (Phe, Tyr, Trp) are transported by separate  $H^+$ -linked mechanisms in *L. lactis* [16,87,123–126]. Transport of branched chain amino acids driven by  $\Delta p$  has also been demonstrated for *Lc. mesenteroides* subsp. *dextranicum* [127]. In *E. hirae* and *Streptococcus pyogenes*, unlike *L. lactis*, the neutral amino acids glycine, alanine (or  $\alpha$ -aminoisobutyric acid), serine and threonine are transported by a common  $\Delta p$ -driven system [128,129]. Evidence, often based on metabolic inhibitor studies, points to  $H^+$ -linked uptake of these amino acids in organisms like *S. thermophilus*, *S. agalactiae*, *S. pneumoniae* and others (for review, see Konings et al. [16]).

The  $H^+$ -linked amino acid transporter that has been analysed in depth is the branched-chain amino acid carrier of *L. lactis* [125,126,130–134]. The substrate specificity, the kinetic mechanism of transport, the role of the lipid environment, and some other molecular properties of the partially purified protein reconstituted into proteoliposomes have been analysed. Several properties of the system have been reviewed in the past [16,87].

In recent years important progress has been made in the study of the peptide transporters of lactic acid bacteria. Whereas peptides of three upto at least six residues are taken up by an ATP-driven binding lipoprotein-dependent transport system (OppFDCBA) [72,75], di- and tripep-

tides are taken up by an  $\Delta p$ -driven transport protein (DtpT) in *L. lactis* [135]. So far, the DtpT and OppFDCBA peptide transporters together with the proteinase (PrtP) are the only components of the proteolytic system of *L. lactis* that are essential for casein utilization and for growth in milk [75,136,137]. The di/tripeptide transport system also represents the first demonstration of  $H^+$  coupled peptide transport in bacteria. The gene encoding the di/tripeptide transporter (DtpT) has been isolated, sequenced and functionally expressed in *E. coli* (A. Hagting, B. Poolman and W.N. Konings, unpublished results). DtpT is a highly hydrophobic protein with a unique primary structure which has no significant similarity to proteins in the MIPS protein data base (release Dec. 1992). The secondary structure of the DtpT protein most likely consists of 12 transmembrane  $\alpha$ -helical segments which span the membrane in a zig-zag manner. Using the 'positive inside' rule [138], the amino- and carboxy-termini are present on the outside of the cytoplasmic membrane unlike most secondary transport proteins [139].

The plasmid-encoded citrate transport gene (*citP*) of *L. lactis* subsp. *diacetylactis* has been isolated and functionally expressed in *E. coli* [140]. The CitP protein is homologous to the citrate- $H^+$ - $Na^+$  symporter (CitS) of *Klebsiella pneumoniae* [141]. For the CitS protein of *K. pneumoniae* it has been shown that dianionic citrate ( $H$ -Cit $^{2-}$ ) is transported in symport with 1  $Na^+$  and at least 2  $H^+$  [142]. Despite the homology between CitP and CitS (overall similarity of 76%), there are no indications that CitP of *L. lactis* uses  $Na^+$  as additional coupling ion.

### Ion-linked cation transport

Bacteria keep their intracellular  $Ca^{2+}$  and  $Na^+$  concentrations well below that of the surrounding medium as long as metabolic energy is available.  $Ca^{2+}$  extrusion in lactic acid bacteria is driven either by ATP [110] or  $\Delta p$  [143]. In fact, for *L. lactis* it has been shown that subsp. *lactis* uses a  $Ca^{2+}$ -ATPase [111] while subsp. *cremoris* excretes  $Ca^{2+}$  by  $Ca^{2+}/H^+$  antiport [143]. Sodium extru-

sion in bacteria is generally attributed to  $\text{Na}^+/\text{H}^+$  antiport mechanisms [144]. The sodium gradient formed can serve as a driving force for, for instance, transport systems catalysing  $\text{Na}^+$ -solute symport ([139]; e.g. alanine, glycine and serine, threonine transport in *S. bovis*, Table 2). In addition,  $\text{Na}^+/\text{H}^+$  antiporters are thought to be involved in the regulation of the cytoplasmic pH [144,145]. In case of the lactic acid bacteria,  $\text{Na}^+/\text{H}^+$  antiport activity has only been demonstrated in *E. hirae* [118,146]. The secondary  $\text{Na}^+/\text{H}^+$  antiporter of *E. hirae* is expressed constitutively, in contrast to the ATP-driven  $\text{Na}^+/\text{K}^+$  exchange system (KtrII) of *E. hirae* which is induced in media with high concentrations of  $\text{Na}^+$  [116] (see above).

### Concluding remarks

The present manuscript updates our 1989 review [16]. Research related to energy-transducing processes in lactic acid bacteria still focusses on two organisms, i.e. *L. lactis* (formerly *Streptococcus lactis* and *S. cremoris*) and *E. hirae* (formerly, *Streptococcus faecalis*), although work on other species is beginning to emerge. In the last 5 years a number of genes encoding transport proteins from lactic acid bacteria have been isolated, characterized and functionally expressed in homologous and heterologous host systems. This has allowed detailed analysis of the molecular properties of these systems both in vivo and in vitro. In this respect research on the lactose transport protein (LacS) of *S. thermophilus* and the lactose PTS of *L. lactis* is most advanced. The recent cloning and characterization of the genes encoding the two peptide transporters of *L. lactis* (DtpT and OppFDCBA), and establishment of their essential roles in the utilization of casein(-derived peptides), are of eminent importance for the unraveling of the 'pathway(s)' of casein breakdown in lactic acid bacteria.

The discovery of precursor/product exchange reactions in bacteria, such as sugar phosphate/phosphate, arginine/ornithine, lactose/galactose, malate/lactic acid, histidine/histamine exchange and others, has its origin for the greater

part in the research on lactic acid bacteria. Similar systems as well as the chemiosmotic circuits associated with the decarboxylation reactions have also been demonstrated in other bacteria.

For all these studies a variety of techniques has been developed, ranging from protocols for the isolation of membrane vesicles, membrane fusion and reconstitution techniques, to the isolation of mutants either by random or targeted mutagenesis (integration) and efficient (electro) transformation protocols. Most of these tools have been developed for *L. lactis* (and *E. hirae*), but appear applicable in one way or another to many lactic acid bacteria.

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